UNIVERISTY OF GONDAR, COLLEGE OF MEDICINE AND HEALTH SCIENCES, SCHOOL OF PHARMACY, DEPARTMENT OF PHARMACOLOGY

IN VITRO EVALUATION OF ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF THE LATEX, GEL, AND 80% METHANOL GEL EXTRACTS OF THE LEAVES OF *ALOE MACROCARPA* TOD (ALOACEAE)

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DEPARTMENT OF PHARMACOLOGY, SCHOOL OF PHARMACY, COLLEGE OF MEDICINE AND HEALTH SCIENCES, UNIVERSITY OF GONDAR APPROVAL SHEET FOR MSC THESIS RESEARCH

As member of examining Board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by Ashenafi Genanew entitled ***in vitro*** **evaluation of antibacterial and antioxidant activities of the latex, gel and 80% methanol gel extracts of the leaves of *Aloe macrocarpa* Tod (Aloaceae)** and recommended that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Pharmacology.

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Final approval and acceptance of the thesis is contingent upon the submission of the final copy of the thesis to the University of Gondar through the Department Pharmacology of the candidate's major department. I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling thesis requirement.

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LIST OF ACRONYMS

AST: Antibacterial susceptibility test

DMSO: Dimethyl sulphoxide

DPPH: 1, 1- diphenyl- 2- picryl hydrazyl

MBC: Minimum bactericidal concentration

MIC: Minimum inhibitory concentration

ROS: Reactive oxygen species

ZI: Zone of inhibition

ABSTRACT

Back ground: Infectious diseases are becoming threats to public health globally and oxidative stress has been implicated in the pathophysiology of various diseases. The use of plants for the welfare of human being has long history. The exudates of the leaf of *Aloe macrocarpa* Tod have been used for the treatment of wounds and chronic diseases in North Gondar. The aim of this study was to investigate antibacterial and antioxidant activities of the latex, gel, and 80% methanol gel extracts of *Aloe macrocarpa* Tod.

Methods: The latex and gel of *Aloe macrocarpa* Tod were collected in sterile way and dried at 40^{o}C in oven. The dried crude gel was further extracted using 80% methanol. The extracts were evaluated for their *in vitro* antibacterial and antioxidant effects. Antibacterial test was conducted against three Gram positive and four Gram negative bacteria by agar well diffusion and macro-broth dilution methods; antioxidant activity evaluation was conducted by DPPH assay. Preliminary phythochemical analysis of all extracts and acute toxicity tests of the latex and gel were conducted using standard methods.

Results: The three extracts showed different degrees of inhibitory activities against tested bacteria at 2, 1 and 0.5 mg/ml in a dose dependant manner and the results were statistically significant ($P \leq 0.05$). Zone of inhibition ranged from 0.00 to 23.00±0.577 mm for all bacteria. The minimum inhibitory concentration values ranged from 31.25- 4000, 31.25 - 2000 and 62.5-8000 μg/ml for latex, 80% methanol gel extract, and crude gel, respectively. The lowest minimum bactericidal concentration was 31.25 μg/ml against *Salmonella typhi* by latex. Latex revealed the greater antioxidant activity with IC_{50} value of 15.5 μg/ml followed by 80% methanol gel extract (19.5 μg/ml) and crude gel extract (36 μg/ml). Phytochemical analysis indicates the likely presence of alkaloids, flavonoids, phenols, tannins, terpenoids, steroid, anthraquinones and saponins. Besides, no sign of toxicity or mortality was observed in mice at 2000 mg/kg.

Conclusion: These results support the use of the plant in the treatment of wounds and chronic diseases associated with oxidative stress; however, further studies are required as to the nature of active compounds responsible for the effects observed.

Key wards: - *Aloe macrocarpa* Tod, Antibacterial activity, Antioxidant activity

1. INTRODUCTION

1.1. Back ground

Beginning ancient times plants have been indispensable sources of both preventive and curative traditional medicine preparations for human beings as well as livestock (1). Medicinal plants continue to play central roles in the healthcare system of large proportion of the world's population. Mainly this is true for developing countries, where herbal medicine has a long and uninterrupted history of use. Recognition and development of medicinal and economic benefits of these plants are increasing in both developing and developed nations (2, 3).

Plants are valuable source of medicines and despite remarkable progress in synthetic organic chemistry, more than one quarter of the prescribed drugs in industrialized countries are derived directly or indirectly from plants (2, 4). They also play an important role in maintenance of human health, management of various ailments and nutrition for the African communities. In developing countries including Africa, most of population relies on plant based preparations in their traditional medicinal system (4).

In Ethiopia, plant remedies are still the most important and sometimes the only sources of therapeutics for nearly 80% of population. Estimated floras of 6500 to 7000 species of higher plants are medicinally important, of which 12% are endemic to Ethiopia (1, 5). More than 95% traditional medicinal preparations in the country are plant origin. The use of herbs for remedies represents not only part of the struggle of the people to meet their essential drug needs but also it is an integral component of the cultural beliefs and attitudes. Antimicrobial and wound healing plant products are among remedies that are commonly available in the markets (6).

Different survey done in the Northern part of Ethiopia on the use of plants for traditional medicine showed that plants are used for treating different ailments. Studies conducted in Gemeda district, East Tigray and North Gondar of Amhara regions indicates the usage of different plants for antibacterial effect and treatment of chronic disease (5, 7, 8).

Recent studies demonstrated that many chemical compounds found in plants have antimicrobial, antioxidant, immune stimulant, hormonal metabolism modulating, anti-hypertensive and anticancer properties (9, 10).

Infectious diseases are the leading cause of death worldwide (10). According to the World Health Organization, about one fourth of the total 57 million annual deaths that occur worldwide are caused by pathogenic microorganisms; this proportion is significantly higher in the developing world (11-13). In addition, infectious diseases remain the main causes of the high morbidity in developing countries where the majority of rural people has limited access to formal or adequate health services and thus heavily recourses to traditional healers (14).

Antioxidants are compounds that can delay, inhibit or prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress (15). Oxidative stress occurs when the generation of free radicals or reactive oxygen species (ROS) exceeds the antioxidant capacity (16). It involves in chronic diseases such as cancer, atherosclerosis, diabetes, inflammation; antioxidant supplements are vital to combat oxidative damage (15-18).

1.2. Statement of the problem

Natural products derived from plants have medicinal, pharmaceutical and industrial applications. Such compounds have modulated several physiological changes in humans and contributed to the promotion of health (19). However, searching of plants as source of potential candidate for drug development is still unsound. Potentially used only 1-10% of plant species available on earth (10).

After centuries of empirical use of herbal preparation in the world, the first isolation of active principles alkaloids such as morphine, quinine in the early 19th century marked a new era in the use of medicinal plants and beginning of modern medicinal plants research. But emphasis shifted away from plant derived drugs with the tremendous development of synthetic pharmaceutical chemistry and microbial fermentation after 1945. In the first half of the 21st century proven and used more powerful and potent synthetic drugs. However, due to the numerous side effects of these drugs, the value of medicinal plants is being rediscovered and some have proved to be as effective as synthetic medicines (9). But still herbal uses continued by majority of traditional practitioners which have no scientific basis including Ethiopia (2, 20).

Even in the age of combinatorial chemistry, natural products have an important place in pharmaceutical development and much more successful than artificially designed compounds. Exploitation of local raw materials by pharmaceutical and allied industries for drug production and conversion to materials of daily uses may be viable approach to reduce dependence on imported drugs specially for developing countries (19). Still there is no official standard and/or local monograph which is the major problem for herbal medicine practices (2).

Diseases due to pathogenic microorganisms including bacteria represent a critical problem to human health. The evolution of multiple drug resistant human pathogenic microorganisms has driven the search for new sources of antibacterial substances, including plant metabolites (21). Investigate efficacy of plant based drugs in traditional medicine has been paid great attention because these drugs elicit few or no side effects, cheap and easily available (9, 21). Additionally, antibiotics are once considered the universal answer to infectious disease, are now known to have a limited effective life span. This is now a common global problem posing enormous public

health concerns (22). Whereas, plants serve as rich, natural and safer sources of antimicrobials may be overcome this problems (14, 17).

Wound infections are most common in developing countries because of poor hygienic conditions. *Staphylococcus aureus* (*S. aureus*), *Streptococcus pyogenes* (*S. pyogenes*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Streptococcus pneumoniae* (*S. pneumoniae*), *Klebsiella pneumoniae* (*K. pneumoniae*) are some important organisms causing wound infection; ROS are deleterious to wound healing process due to the harmful effects on cells and tissues (23, 24). A wide range of antibiotics is being used at present time for treating wound infections but they are now proved to have adverse effects in the human body and developed resistance to these pathogen (23). Therefore, systematic indigenous knowledge gathering on the utilization of known plants among communities may result in the discovery of novel and effective compounds (4).

Highly ROS are present in biological systems from a wide variety of sources (18). Although, almost all organisms possess antioxidant defense and repair systems; these systems are insufficient to cope over entire damage. So, antioxidant supplementation is a promising mean to strengthen the antioxidant defense and repair systems. However, most commonly used synthetic antioxidants such as butylatedhydoxyanisole, butylatedhydoxytoluene and propylgallate have health hazardous like liver damage and carcinogenesis due to this antioxidants from natural source highly recommended (16). Further, natural antioxidants are in high demand for application as pharmaceutical and as food additives at present time (17).

Over all, due to the emergence and spread of disease which results in the impairment of immune system such as HIV and diabetes (25); discovery of new and novel antibacterial and antioxidants have been endless activities for pharmaceutical industries and research institutes. In line with this extensive and misuse of antibiotics and their consequences in drug resistance, make human pathogens become the future challenge for treating infectious diseases. Therefore, efforts should be made to preserve drugs at hand and search for antibacterial as well as antioxidants agents from traditional medicinal plants (26).

1.3. Pharmacology of antibacterial

Substances with anti-infective potential have been applied medically for thousands of years. Although the discovery of effective agents to prevent and treat infections caused by bacteria and other pathogenic microorganisms is one of the most important developments of modern medicine, the use of such agents is not limited to the present era (27).

Currently available antibacterial drugs are numerous and difficult to describe all of the characteristics and clinical applications in this paper. But generally they can be classified based on spectrum of activity, types of action, chemical structure and mechanism of action (27, 28). Ciprofloxacin is second generation fluoroquinolone that blocks bacterial deoxyribonucleic acid synthesis by inhibiting bacterial topoisomerase II and topoisomerase IV. It possesses excellent Gram negative activity and moderate to good activity against Gram positive bacteria (28).

In modern medical practice, the alarming worldwide incidence of antibiotic resistance causes an increasing need for new compounds that can act either by a direct antimicrobial activity or by interfering resistance mechanisms of microorganisms (14). At least eight distinctive mechanisms of antibacterial resistance have been described in bacteria. Enzymatic alteration, decreased permeability, efflux, alteration of target site, protection of target site, over production of target, by pass inhibited process and bind up antibacterial (27).

1.4. Medicinal plants as antibacterial and/or antioxidant agents

Different studies conducted in Africa using different solvent and methods such as stem bark of *Pteleopsis hylodendron* (in Cameroon) (29), root barks of *Cordial gilletii* (in Congo) (14), root of *Ritchiea longipedicellata* (in Nigeria) (2) and different parts of *Curtisia dentate* (in South Africa) (17) showed that different degrees of antioxidant and antimicrobial activities. Among these, stem bark of *Pteleopsis hylodendron* revealed dose dependent ZI which are statistically significant ($p \leq 0.05$) at 200, 100 and 50 mg/ml (29).

Similarly, studies carried in Ethiopia on methanol (80%) extracts of different parts of plants such as *Clerodendrum myricoides, Satureja punctata, Urtica dioica, Ajuga remota, Gnidia stenophylla, Ficus plamata, Grewia ferruginea* and *Periploca linearifolia* displayed various degrees of antibacterial and antioxidant activities (18, 30).

1.5. The genus of *Aloe*

The term aloe is derived from the Arabic word alloeh, meaning 'shiny bitter substance' in reference to the exudates (31). The genus *Aloe* which is represented over 500 species (32, 33).

They are native to main land Africa and small islands in Indian Ocean except few species occurring in Arabian Peninsula. Also, high *Aloe* diversity occur in Madagascar, East and West Africa (31). The flora of Ethiopia and Eretria possess 46 species of *Aloe*, out of which 41 are reported to be endemic. Only five species: *A. lateritia*, *A. macrocarpa*, *A. rivae, A. secundiflora* and *A. vituensis* are wide spread extending to East and West Africa (34).

Study done from 1996 to 1999 in the Simen Mountains indicate presence of *Aloe* species in Northern Gondar, as this report *A. macrocarpa* and *A. pulcherrima* are the new recorded *Aloe* species of flowering plants for Gondar floristic region of Ethiopia (35).

Members of this genus have been known in medicinal use, commerce and horticulture (34). Today *Aloes* are renowned worldwide as a source of natural products (31). Different *Aloe* species would have various chemical contents, and health benefits (36).

1.5.1. Traditional uses

At least 25% of *Aloe* species are valued for traditional medicine (33). Their use was recorded by the Egyptians, Assyrians and Mediterranean peoples as far back as 1500 B.C.; the Greek physician Dioscorides was the first to describe the use of *Aloe* to treat mouth infections, sores, wounds and as a purgative. Still *Aloes* are a popular folk medicine among peoples of Indian, Chinese and Mexican (32).

They are also harvested and utilized for traditional medicine in numerous African countries and nearly 60 species are used in Southern Africa alone for treating different aliments (31). In some countries of East Africa *Aloes* use also documented, from Kenya up to half of species available in the country used as medicine mainly for treatment of malaria, when coming to specific species *A. lateritia* gel used as beauty therapy and *A. ruspoliana* used for killing hyenas and wayward dogs in Kenya (37). *A. microdonta*, to treat jaundice and skin diseases in Somali (38). In Ethiopia *A. vera* for diabetes (7), *A. harlana* for the treatment of infectious and inflammatory diseases (39) and *A. otallensis* for the treatment of malaria (40).

1.5.2. Pharmacological activities

Leaves of *Aloes* are the source of two products that are quite different in their therapeutic properties, aloe latex and aloe gel (31, 32). These two products are obtained from two different specialized cells, gel from the parenchymatous cells in the inner leaf and latex from pericyclic cells in the margins of the leaf (32).

Gel part is clear jelly-like substance, first used clinically in the 1930s for the treatment of radiation burns. Today it is a familiar ingredient used in ointments and cosmetic industries (32) and widely recognized herbal remedy to relieve thermal burn, sunburn and promote wound healing (21, 41). Whereas the latex is mainly used for laxative effect (32) but study carried in Ethiopia from latex and the two isolated compounds (anthrone and chromone) of *A. harlana* showed antimicrobial and antioxidant activities (39).

The genus has been known to contain many species that demonstrated diverse pharmacological activities, such as antimicrobial, antioxidant, anti-diabetic, hepatoprotective, anti-inflammatory, wound healing, anticancer, anti-malaria and toxicity effects (20, 32, 42, 43).

1.5.2.1. Antimicrobial activity

Extractions of *A. vera* by different solvent, *in vitro* investigated antibacterial and antifungal activities against the human pathogens (21, 43, 44). The antibacterial activity by disc diffusion, dimethyl sulphoxide (DMSO) *A. vera* gel extracts at dose of 100, 200 and 400 μg/ml zone of inhibition (ZI) by mm showed *Bacillus subtilis* (7, 8 and 10), *E. coli* (10, 12 and 13), *S. aureus* (8, 9 and 10.5) and *Proteus vulgaris* (10, 11 and 12) respectively but not effect on *S. typhi* (44).

The root extracts of *A. vera* both the aqueous and methanolic extracts showed various degree of antibacterial activities against *in vitro* tested bacteria. By agar well diffusion method (100 μl of the extracts) against *Bacillus cereus*, *E. coli*, *P. aerugenosa* and *Enterobacter aerogens*; methanol extract, ZI was investigated between 7 - 20 mm at100 μg/ml whereas water extract was 2-4 mm. The minimum inhibitory concentration (MIC), the methanol extract was between 10 and 25 μg/ml and aqueous extract ranged from 25 – 60 μg/ml. The minimum bactericidal concentration (MBC) for methanol extract ranged between 20 and 50 μg/ml and aqueous extract ranged from 50 to 120 μg/ml (42).

1.5.2.2. Antioxidant activity

In vitro antioxidant activity of *A. vera* gel has been reported using oxygen radical absorbance capacity and ferric reducing antioxidant power methods (36). Another study conducted on latex of *A. otallensis* (in Ethiopia*)* exhibited radical scavenging activity with an IC_{50} (decrease the initial DPPH absorbance by 50%) value of about 26.9 μg/ml by DPPH assay (40).

The reported review of *A. ferox* describes, it has been antioxidant activity by using oxygen radical absorbance capacity, ferric reducing antioxidant power and DPPH assay (45). Also, *in vivo* study conducted on 95% ethanol *A. vera* leaf gel extract in male albino rats showed significant antioxidant effect (46).

1.5.2.3. Adverse effects/toxicity

A study conducted in Japan to evaluate the chronic toxicity of *A. arborescens*, the results revealed that the plant extracts did not possess any adverse effect in rats which were treated daily with 87.7 mg/kg/day and 109.7 mg/kg/day for male and female, respectively (47).

In humans, there are no published controlled toxicology studies *in vivo* but frequently reported to *Aloe* preparations are allergic conditions and hypersensitivity (32, 45). However, as general advised pregnant women should not take *Aloe* latex because of its cathartic action, which may cause severe uterine contractions and increase the risk of miscarriage and also, nursing mothers because of the possibility of causing severe cramps and diarrhea in infant (32).

1.5.3. Phytochemistry

The levels and types of chemical continent in *Aloe* plants are highly variable according to species and strain, as well as growth conditions (48). The gel consists water (mainly) and active compounds, including flavonoids, saponins, sterols, vitamins, phenolic compounds, etc (43).

Study carried out in Iran from leaf gel and 95% ethanol leaf gel extracts of *A. vera revealed* presence of polyphenols, sterols, indoles, alkaloids, etc (36). Also, current reports of *A. vera* gel has been identified more than 75 active ingredients such as flavonoids, saponins, sterols (48). Study conducted in Nigeria from ethanolic extracts of leaf of *A. perryi* showed the presence of

tannins, saponins, and flavonoids but not alkaloids (49) and different investigation of *A. ferox* extensively revealed the presence of chromones, anthraquinones, and phenolic compounds (45).

1.6. *Aloe macrocarpa* Tod.

A. macrocarpa Tod (known as "Eret" Amharic; by oral communication in Kolla Diba) is one of species from family Aloaceae. It had been described by Italian botanist Agostino Todaro from a specimen collected in Ethiopia (probably from Tigray region) and by German botanist Georg H. W. Schimper in 1870 (41, 50).

Today, it is known to have a wider distribution extending east to west Africa: Ethiopia, Eritrea, Djibouti, Somalia, Sudan, Kenya, Burkina Faso, Mali and Nigeria (34, 41, 50, 51) and the documents indicated that the term 'macrocarpa' drive from macro- large and carpa – fruit, meaning "large fruit size" (41) and it's morphology is shown in Figure -1.

It is a member of genus *Aloes* known as the 'saponaria' group which have soft and spotted leaves and a basal swelling of the perianth tube. However, it is clearly distinguished from the other Ethiopian members of the group by the numerous pale spots on the leaves, which also have distinct darker longitudinal lines, the perianth with a markedly globose basal swelling and the large capsule. It is rosettes, stemless, rarely developing short erect stems in shaded sites, solitary or forming small groups. Grows in open grassland, often between clusters of evergreen bushes, on rocky slopes to plateau areas with darker soil between 1400 and 2200 meter and October to April is main flowering period in Ethiopia, its size varies from site to site for example from high altitudes in Arsi region in Ethiopia are much smaller than plants growing at lower altitudes (41).

Methanolic leaf extracts of *A. macrocarpa* Tod (collected from Ethiopia, Adamitulla) was found to contain flavonoids (isoorientin and isovitexin) (33).

In North Gondar the leaf exudates of *A. macrocarpa* Tod is used as traditional medicine for treating wound, for chronic diseases, to protect from evil spirit, cessation of breast feeding and for replacement of hair loss from cattle (by oral communication).

Figure - 1:- Morphological view of *Aloe macrocarpa* Tod.

1.7. Models for antibacterial and antioxidant test

1.7.1. Models for antibacterial test

Novel antibacterial agents searching from higher plants has been a great interest in the last few decades, but results generated from many of these studies cannot be directly compared due to lack of standardization in particular methods employed. Due to this many studies reported in the literature describing different methodologies (52). But, generally models may be divided in to two: *in vitro* antibacterial susceptibility test (AST) and *in vivo* AST (53-55).

1.7.1.1. *In vitro* antibacterial susceptibility test

It is the most common method, involves measuring the antibacterial activities against the test bacteria by determining the ZI and/ or MIC and/or MBC (42, 53). Currently employed *in vitro* evaluation of plant extracts are diffusion and dilution methods (52, 53).

1.7.1.1.1. Diffusion method

It is commonly described as qualitative test (27). Disc diffusion and agar well diffusion are the two methods of diffusion, most commonly used screens to determine AST. The diffusion of an antibacterial of a specified concentration from disks, tablets or strips into solid culture medium is termed as disc diffusion and diffusion through the holes punched in the agar medium containing culture, termed as agar well diffusion, both methods are mainly based on determination of an inhibition zone (56).

1.7.1.1.2. Dilution methods

It is commonly termed as quantitative test (27), includes broth and agar dilution methods. Both can be used to determine the lowest concentration of the assayed agent that inhibits the visible growth of the bacterium being tested (54).

Broth dilution is a technique in which a suspension of bacterium is tested against varying concentrations of agents (usually serial two fold dilutions) in a liquid medium of documented formulation. It can be performed either in tubes containing a minimum volume of 1 ml (macro-dilution) or in smaller using micro-titration plates (micro-dilution) (54, 57).

MIC- is the lowest concentration of the agent that inhibits visible growth of bacteria, usually after 18 – 24 hours incubation period. MBC- is the lowest concentration of an antibacterial agent that kills greater than or equal to 99.9% the test inoculums after incubation (usually 18 to 24 hours) (27). MBC testing is an accepted parameter in the evaluation of new antimicrobial agents and used as a research tool (29, 56). Following performance of a conventional broth dilution MIC susceptibility test tubes or wells containing concentrations of antibacterial agent equal to or greater than the MICs are sub-cultured to determine whether the initial inoculums is inhibited from multiplying (static action) or killed (bactericidal action) (29, 42).

1.7.1.2. *In vivo* antibacterial susceptibility test

This technique is not frequent, usually applied when the plants extracts/novel agents showed excellent *in vitro* antibacterial activity therefore before clinical studies performed to check the toxicity and *in vivo* efficacy of such agents (55, 58). In this model the laboratory animal (mouse, rabbit, chicken, etc) can be systemically infected (by exposing microorganism using appropriate routs) and /or local infected (commonly skin burn). Then observe clinical signs such as fever, dullness, depression, diarrhea, ruffled feathers, refusal of feeding; depending on bacterial proprieties and design models considered as infected then give the design plant extract/novel agent to demonstrate antibacterial effect (55, 58).

1.7.2. Models for antioxidant test

Oxidation is a metabolic process that leads to energy production necessary for essential cell activities. However, it may also leads to the unavoidable production of ROS, which are involved

in the onset of many diseases (24). The ROS can exist in living organisms either endogenous or exogenous sources (59). Models used for conducting antioxidant activity of herbal preparations may broadly be grouped in to two: *in vitro* and *in vivo* models (24, 59, 60).

1.7.2.1. *In vitro* models

This method detects the scavenging ability of plant extracts to ROS, such as hydroxyl radical, super oxide anion radical, hydrogen peroxide, single oxygen, nitric oxide radical, hypochlorite radical (59). The *in vitro* methods (models) that measure these many compounds due to this also measuring techniques are numerous, for sample:- DPPH, ferric reducing ability of plasma, thiobarbituric acid, hydroxyl radical, nitric oxide, super oxide radical assays, etc (59, 60).

1, 1- diphenyl- 2- picryl hydrazyl (DPPH) Method

It is the most widely used method for screening of antioxidant effects of plant extracts (18, 59). The molecule of DPPH is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole means as it is as radical state, methanolic solution of this compound is violet color when it reacts with an antioxidant it is reduced to the molecular form (DPPHH) which is yellow color (61).

DPPH assay is the procedure that involves measurement of decrease in absorbance of DPPH at its absorption maximum of 517 nm, which is proportional to concentration of free radical scavenger added to DPPH reagent solution (18, 59).

1.7.2.2. *In vivo* model

Free radicals attack the unsaturated fatty acids of bio-membranes, which results in lipid peroxidation and destruction of proteins and DNA, which causes a series of deteriorative changes in the biological systems leading to cell inactivation. The antioxidants may act by raising the levels of endogenous defenses by up-regulating the expression of genes encoding the enzymes such as superoxide dismutase, catalase, or glutathione peroxidase (24) and by decreasing levels of lipid peroxidation and hydroperoxides (46). *In vivo* antioxidant activity can detect by measuring the above measurements and also may examine histopathology of organs in laboratory animal that oxidative stress is induced by chemical such as carbon tetrachloride, streptozotocin, alloxan (24, 46).

1.8. Significance of the study

Infectious diseases are the main cause of morbidity and mortality in developing countries. Antimicrobial resistance is an emerging threat to global health; this causes an increasing need for new compounds that can act either by a direct antimicrobial activity or by interfering resistance mechanisms of microorganisms. Medicinal plants represent a valuable source for this kind of compounds and play an important role in the prevention and/or treatment of bacterial infection as well as chronic disease involves oxidative stress especially in developing countries where resources are limited.

It has been claimed that about 80% of the Ethiopian population rely on traditional medicinal plants for treating various illnesses including bacterial infection and chronic diseases. In Northern Gondar *A. macrocarpa* Tod used for treating wounds and chronic diseases without scientific base.

Therefore, it is with this background, the study was conducted to evaluate the antibacterial and antioxidant activities of *A. macrocarpa* Tod.

2. OBJECTIVES

2.1. General objective

- This study was used to test the antibacterial and antioxidant activities of the latex, gel and 80% methanol gel (hydroalcoholic gel) extracts of *Aloe macrocarpa* Tod.

2.2. Specific objectives

- To carry out qualitative phytochemical screening of the extracts
- To find out antibacterial effects by zone of inhibition, MIC and MBC of the extracts
- To determine antioxidant capacity of the extracts by DPPH assay
- To determine acute oral toxicity of latex and gel extracts

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Plant material

Latex and gel (from leaves) of *A. macrocarpa* Tod were collected in December 2013 near Kolla Diba town, North Gondar zone of Amhara national region, 765 kilometer away from Addis Ababa the capital city of Ethiopia. The specific site of collection is Ambo Gedle, one kilometer away North East direction from Koll Diba Town. The plant was authenticated by the National Herbarium, Department of Biology, Addis Ababa University and a voucher specimen (number 08768) was deposited.

3.1.2. Chemicals and reagents

The following chemicals and reagents were used for this study: methanol, hexane and ethyl acetate (Unichem laboratories- pharmaceutical company, India), DMSO, ammonia and ferric chloride (Avishker international Pvt, India), ciprofloxacin 500 mg tablet (Cadila Pharmaceuticals PLC, Addis Ababa-Ethiopia), ascorbic acid 500 mg tablet (Ethiopian pharmaceuticals manufacturing factory, Addis Ababa-Ethiopia), 0.5 Mc Farland Turbidity (Sparks, USA), DPPH (Sigma Aldrich, Germany), Mueller Hinton agar, nutrient agar, nutrient broth and blood agar (Oxoid Ltd, Hampshire-England), mercuric chloride, acetic acid, ethanol, iodine and potassium iodide (Supertek international Pvt, India), acid alcohol, hydrochloric acid and benzene (Nice, Cochin-India), chloroform, glacial acetic acid and sulfuric acid (Avantor performance materials international Pvt, USA) and aluminium chloride anhydrous (BDH chemicals Ltd, England). All the chemicals and reagents used in this study was analytical grade. Most of them are purchased from Wabir pharmaceutical and medical equipment trade PLC, Bahir-Dar – Ethiopia and Gondar Hospital while the rest received from different departments of pharmacy and/or microbiology department, University of Gondar.

3.1.3. Instruments and medical supplies

The following instruments and medical supplies were used for this study: UV-Visible-spectrophotometer (Henan Hi-Tech Instruments Co. Ltd, China), oven (model-GP130/CLDA/250/HYD, Leader engineering-St Helen, USA), Whatman no-1 paper18cm

diameter and 0.1µm pore diameter (Whatman Ltd, England), refrigerator (Hitachi, Tokyo-Japan), incubator (DHP-9052, Hangzhou Chincan Trading Co. Ltd., China), electrical balance (AB-104, China), autoclave (Dixons surgical instruments Ltd, England), class II safety cabinet (Beaker company, USA), shaker (HY-5A, China), electrical stove (Simfer, Turkey), Hi antibiotic zone sale-c (HiMedia, India), transfer pipette, plastic cuvette, butcher funnel, syringe with needle, cotton, glove, spatula, applicator sticks, measuring cylinders, flasks, bunsen burner, calibrated loop, cotton swaps, glass test tube, petri dish, beaker, bottle, knife, cork borer, wire loop, mice cage, mice gavage and aluminum foil.

3.1.4. Microorganisms

The test was carried out against three Gram positive bacterial strains: *S. aureus (*ATCC 25923), *S. pneumoniae* (ATCC 49619), and *S. pyogenes* (ATCC 19615) and four Gram negative bacterial strains: *E.coli* (ATCC 25922), *P. aeruginosa* (ATCC 2706), *K. pneumoniae* (ATCC 700603), and *S. typhi* (ATCC 1912/R). All the standard bacterial strains were obtained from Gondar Hospital and department of microbiology, University of Gondar and stored below -4^{o}C until used for this experiment.

Figure - 2:- Pure seven standard cultured bacteria

3.1.5. Animals

Female Swiss albino mice weighing between 24 -30 gram and 8 – 10 weeks of age were obtained from colony in the animal unit of the University of Gondar, College of Medicine and Health Sciences, School of Pharmacy, Department of Pharmacology.

3.2. Methods

3.2.1. Preparation of plant material and extraction procedure

The preparation of the plant materials and the extraction were conducted using standard procedures (36, 43, 44, 62) as described in Figure -3.

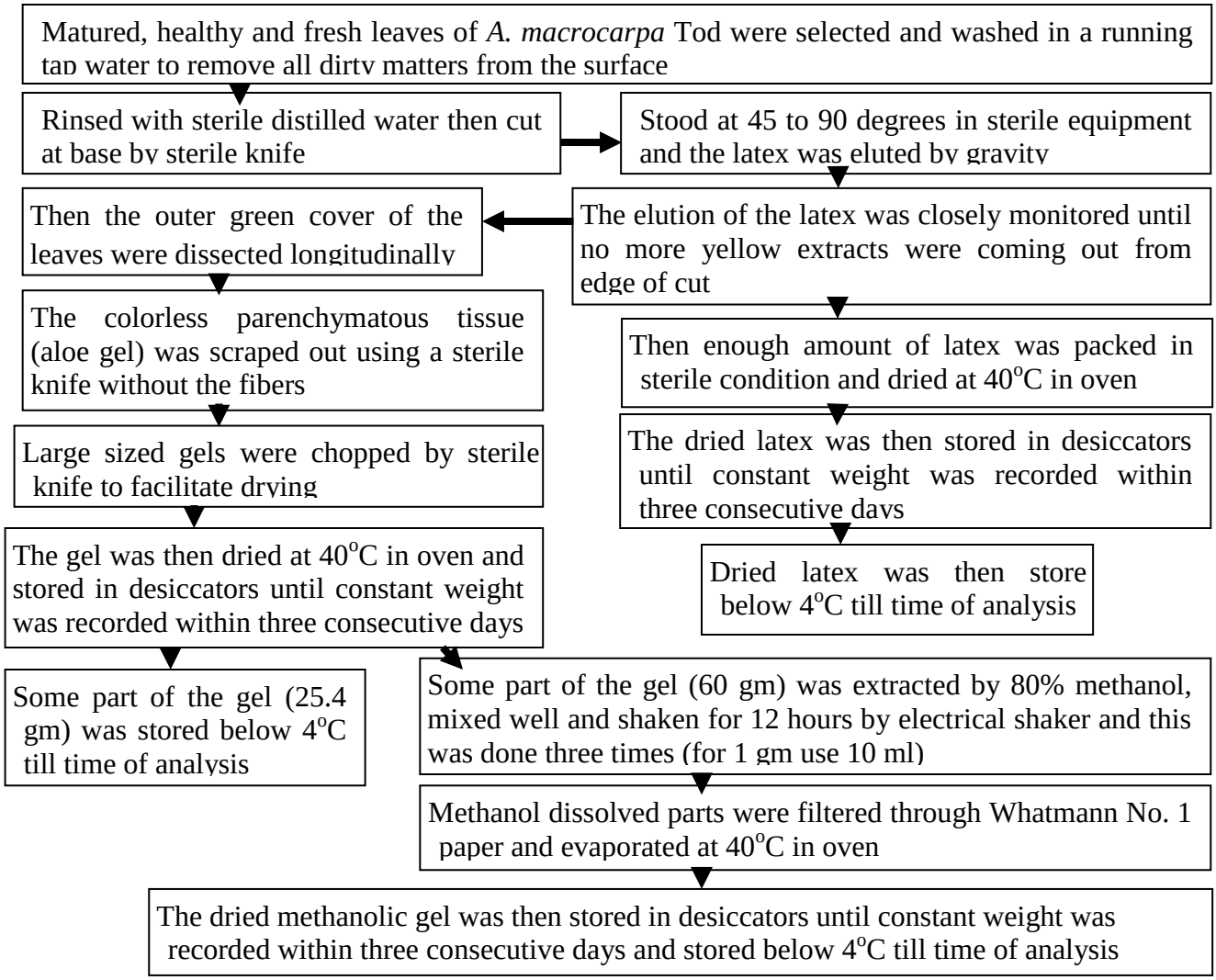

Figure - 3:- Schematic diagram of extraction procedure

3.2.2. Phytochemical screening

Standard qualitative phytochemical analyses were employed to detect the major secondary metabolites such as alkaloids, flavonoides, phenols, tannins, terpinoids, saponins, steroids, anthraquinones and cardiac glycosides as described in (2, 17, 63-66). The specific procedures can be seen from the annex.

3.2.3. Antibacterial effect test

3.2.3.1. Inoculum preparation

Each inoculum of standard bacteria strain was prepared by inoculating a loopful of test bacteria from a colony in 5 ml of nutrient broth and mixed gently until it formed a homogenous suspension. The turbidity was matched with 0.5 Mc Farland standards (57, 67).

3.2.3.2. Antibacterial assay

Antibacterial assays of the extracts were done by the agar well diffusion method described by Kumar, *et al*, 2012 (42) with slight modification. Bacterial broth culture was prepared to a density of 10^8 cells ml^{-1} of 0.5 Mc Farland standards and on each plate, equidistant wells were prepared with a 6 mm diameter sterilized cork borer then randomly label. The aliquot was spread evenly onto Muller Hinton agar using sterilized cotton swab; for fastidious bacteria (*S. pneumoniae* and *S. pyogenes*) additionally used 5% sheep blood (57, 67). Next, 100 µl of each extract at concentrations of 2, 1 and 0.5 mg/ml, by using DMSO as solvent, were applied aseptically into a respective agar wells. Ciprofloxacin 50 µg/ml, dissolved by sterile water, was used as positive control and DMSO (100 µl) was included as negative control. The agar plates were allowed to stand on bench for 30 minutes at room temperature for pre-diffusion and then incubated at 37^oC for 24 hours. Antibacterial activity was determined by measuring the ZI around each well (excluding the diameter of the well by subtracting 6 mm from ZI results). For each extract, three replicate trials were conducted against each organism.

3.2.3.3. Determination of Minimum Inhibitory Concentration (MIC)

The MIC tests were performed for bacteria species responded by diffusion test at any one of tested doses and the estimation of MICs of the extracts were determined by the method described by Kumar, *et al*, 2012 and Asamenew, *et al*, 2011 (39, 42) with some modifications. Briefly, 0.5 ml of varying concentrations of the extracts (ranging from 15.625 µg/ml to 8 mg/ml but for S. *aureus* up to 256 mg/ml was used) were transferred into test tubes containing 5 ml nutrient broth; for fastidious bacteria additionally used 5% sheep blood (57, 67), then10 µl of the test organism was introduced into each tube. The procedure was repeated using the standard drug, ciprofloxacin 0.625 -20 µg/ml. DMSO was the solvent used to dissolve the extracts and

distilled water for ciprofloxacin. A sterility control was also carried out using nutrient broth plus DMSO. Each test and sterility control was incubated aerobically at 37^{o}C for 24 hours. The lowest concentration of the extract that produced no visible growth (turbidity) was recorded as the MIC.

3.2.3.4. Determination of Minimum Bactericidal Concentration (MBC)

The estimation of MBCs of the extracts were determined by the method described by Kumar, *et al*, 2012 (42) with some modifications. The MBC test was determined by taking a loopful sample for each set of test tubes starting from the tube of MIC value and these samples were sub cultured onto a fresh nutrient agar (for fastidious bacteria additionally used 5% sheep blood (57, 67)) then incubated at 37^{o}C for 24 hours and sterility control was also carried out by left sub media without any inoculation. The least concentration of the extracts with no visible growth after incubation was taken as the MBC.

3.2.4. Antioxidant activity test

The antioxidant activities of the plant extracts and the standard were assessed on the basis of the radical scavenging effect of the stable DPPH free radicals. The DPPH test was carried out as described by Asamenew, *et al*, 2011 (39) with some modifications. Fifty micro-liters of various concentrations (ranging 1 – 1000 μg/ml) of the extracts and ascorbic acid (as standard) were mixed separately with 5 ml of 0.004% methanol solution of DPPH, using DMSO as a solvent. The mixtures were incubated for 30 minute at 37^{o}C. After incubation, absorbance of the mixture was read at 517 nm using UV-Visible spectrophotometer (NV-203). Tests were carried out in triplicate and average values were taken.

The percentage radical scavenging activity (%RSA) was calculated using the following formula:

$$\%RSA = [(A_0 - A_s)/A_0] \times 100$$

Where:- A_0 is the absorbance of the control (containing 5 ml of 0.004% methanol solution of DPPH and 50 μl DMSO) and A_s is the absorbance of the test sample.

The IC_{50} (the concentration of the samples that caused 50% inhibition) was determined graphically using Microsoft Excel 2010.

3.2.5. Acute oral toxicity test

Acute oral toxicity test was performed according to OECD guideline 425 (68) which is internationally acceptable. Briefly, ten female mice weighing between 24 -30 gram and 8-10 weeks of age were acclimatized one week prior to dosing. Two mice were taken randomly for sighting study and food but not water was withheld for 4 hours. Following the period of fasting, the animals were weighed and administered 2000 mg/kg from each extract. One mouse received latex and the other received crude gel and they continued fasting for 2 hours. Animals were observed individually after dosing, for the first 24 hours with special attention given during the first 4 hours and daily thereafter for a total of 14 days. The experiment was conducted in accordance with the accepted laboratory animal use.

Observations include changes in skin, fur, eyes, mucous membranes, respiratory, circulatory and autonomic and central nervous system, somatomotor activity and behavior pattern. Attention was directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. As the two mice in the sighting test were not observed for any sign of toxicity for 24 hours, the limit toxicity tests were performed for both extracts.

3.2.6. Data analysis

The data were analyzed using SPSS version 20.0. Means and standard error of mean (SEM) of the triplicate tests were calculated using one-way analysis of variance (ANOVA). The post hoc multiple comparisons Duncan and Tukey were used for antibacterial tests and antioxidant activities, respectively. Results were considered significant when $p \leq 0.05$.

3.2.7. Quality control

To get reliable and valid result, the following quality control measures were taken: avoidance of contamination, checking the reagents or standard bacteria quality before starting procedure, positive and negative controls were used and recording of the results were done properly.

3.2.8. Ethical consideration

This study protocol was reviewed and approved by Ethical Review Board of the Department then ethical clearance was obtained from Pharmacology Department, School of Pharmacy, College of Medicine and Health Sciences, University of Gondar before actual work was commenced.

3.2.9. Pilot study

The pilot study was conducted before the actual experiment to make sure that the designs for fractional gel extraction and antibacterial test were appropriate. The intent of the pilot extraction study was to fractionate the crude gel as per the method described by Thiruppathi, *et al*, 2010 (43) which was employed for *A. vera* gel extraction with some modifications. Thirty grams of crude gel of *A. macrocarpa* Tod was taken and intended to be extracted successively by 80% methanol, ethyl acetate, and n-hexane in a ratio of 1 gm crude gel by 10 ml of solvent. When it was first extracted by 80% methanol as per the aforementioned ratio, the extract was found to be highly concentrated. To make sure efficient extraction by 80% methanol the residue was then extracted two times more. The final methanol extract residue was then re-extracted by ethyl acetate and n-hexane. It was done three times for each solvent. The effort made to extract by the latter two solvents ended up with no quantifiable extracts. For this reason, the main research was done only by 80% methanol.

It was reported that the antibacterial activity of some plant extracts has been done at a concentration of 400 mg/ml (2). For the pilot test stock solutions of the extracts (latex, gel, and 80% methanol gel) were first prepared at a concentration of 400 mg/ml by using DMSO as a solvent. But good solubility for the crude gel was obtained at a concentration of 4 mg/ml. Therefore, the pilot study was done in a range of 4 - 0.25 mg/ml. At a concentration of 0.25 mg/ml no ZI was observed against some tested bacteria. Due to this three consecutive two fold concentration of the extracts were used for main tests, i.e. 0.5, 1 and 2 mg/ml.

3.2.10. Dissemination of the result

The findings of this study will be submitted to department of pharmacology and graduate studies institute of University of Gondar. An attempt will be made to present the findings in different conferences and workshops, publication will be considered on one of the scientific journals.

4. RESULTS

4.1. Percentage yields of extracts

The dried latex, crude gel, and hydroalcoholic gel extracts of *A. macrocarpa* Tod leaf showed yellow-purple, reddish and fluorescent black, respectively. To obtain dried powders, the latex and gel were put inside an oven for 40 and 25 days respectively. The percentage yields of the extracts are shown in Table -1.

Table -1:- Actual and percentage yields of the leaf extracts of *Aloe macrocarpa* Tod

Name of extract	Total collected/used	Actual yield (in gram (g))	Percentage yield
Latex	180 ml	48.62	27.01(w/v)
Gel	8,000 ml	85.4	1.07(w/v)
80% Methanol gel	60 g	33.49	55.82(w/w)

4.2. Phytochemical study

As shown in Table -2, preliminary phytochemical screening of the dried latex, gel, and 80% methanol gel extracts of *A. macrocarpa* Tod by chemical methods revealed the presence of alkaloid, flavonoid, phenol, tannin, terpenoid and steroidal compounds.

Table - 2:- Phytochemical screening result of the *Aloe macrocarpa* Tod leaf extracts

Secondary metabolites		Type of extract		
		Latex	Gel	80% Methanol of gel
Alkaloids	Mayer's test	+	+	+
	Wagner's test	+	+	+
Flavonoids	Ammonium test	+	+	+
	Aluminum chloride test	+	+	+
Phenols		+	+	+
Tannins		+	+	+
Terpenoids		+	+	+
Saponins		+	-	-
Steroids		+	+	+
Anthraquinones		+	-	-
Cardiac glycosides		-	-	-

Key: + = present, - = absent

4.3. Antibacterial activity

In this investigation, the antibacterial activity of the latex, gel, and hydroalcoholic gel extracts of the leaf of *A. macrocarpa* Tod were evaluated using agar well diffusion and macro-broth dilution methods. Tables -3 and 4 as well as Figure -4 show the antibacterial activities of the extracts against seven standard bacteria.

Antibacterial assay of the three extracts showed various degree of inhibitory activity against tested micro-organism at the concentration of 2, 1 and 0.5 mg/ml. All tested bacteria were responded to the standard drug (ciprofloxacin).The ZI for the latex extract ranges from 0.00 to 23.00±0.577 mm for all bacteria at the tested doses. *S. typhi* was the most sensitive organism (14.00±0.577 -23.00±0.577 mm) towards the latex while *S. pyogenes* was the least sensitive (0.00 mm/no effect) organism. *P. aeruginosa* was the most sensitive organism for both the crude gel and hydroalcoholic gel extracts with ZI 8.00±0.577 to 17.00±0.577 and 12.33±0.333 to 20.67±0.667 mm, respectively; while no effect was observed on *S. typhi*. For all responded bacteria, statistically significant ($P \leq 0.05$) dose dependent effects were seen (Table -3).

Table - 3:- Antibacterial effect (Zone of inhibition in mm) of the leaf extracts of *Aloe macrocarpa* Tod.

Tested bacteria	Dose (mg/ml)	Type of extract			DMSO (100µl)	Ciprofloxacin (50µg/ml)
		Latex	Gel	80% Methanol of gel		
S. aureus ATCC 25923	2(200µg)	8.67 ± 0.667^{e}	6.67 ± 0.333^{cd}	10.33 ± 0.882^{f}	$-^{a}$	19.00 ± 0.577^{g}
	1(100µg)	5.33 ± 0.667^{c}	3.00 ± 0.577^{b}	7.00 ± 0^{d}		
	0.5(50µg)	1.33 ± 0.333^{a}	$-^{a}$	3.33 ± 0.667^{b}		
S. pneumoniae ATCC 49619	2(200µg)	18.67 ± 0.667^{gh}	15.33 ± 0.667^{ef}	20.00 ± 0.577^{h}	$-^{a}$	16.00 ± 0.577^{ef}
	1(100µg)	14.33 ± 0.882^{de}	10.33 ± 0.333^{c}	17.00 ± 0.577^{fg}		
	0.5(50µg)	10.67 ± 0.667^{c}	7.00 ± 0.577^{b}	13.33 ± 0.667^{d}		
S. pyogenes ATCC 19615	2(200µg)	$-^{a}$	10.67 ± 0.333^{d}	13.67 ± 0.882^{e}	$-^{a}$	21.33 ± 0.667^{f}
	1(100µg)	$-^{a}$	5.00 ± 0.577^{c}	9.67 ± 0.667^{d}		
	0.5(50µg)	$-^{a}$	1.67 ± 0.333^{b}	5.00 ± 0.577^{c}		
E. coli ATCC 25922	2(200µg)	18.00 ± 0.577^{f}	13.67 ± 0.333^{e}	17.00 ± 0.577^{f}	$-^{a}$	26.33 ± 0.333^{g}
	1(100µg)	12.67 ± 0.667^{de}	8.67 ± 0.333^{c}	12.00 ± 0.577^{d}		
	0.5(50µg)	8.67 ± 0.882^{c}	5.33 ± 0.333^{b}	7.67 ± 0.333^{c}		
P. aeruginosa ATCC 2706	2(200µg)	19.67 ± 0.667^{f}	17.00 ± 0.577^{e}	20.67 ± 0.667^{f}	$-^{a}$	22.67 ± 0.882^{g}
	1(100µg)	15.00 ± 0.577^{d}	13.00 ± 0.577^{c}	15.33 ± 0.667^{de}		
	0.5(50µg)	12.00 ± 0.577^{c}	8.00 ± 0.577^{b}	12.33 ± 0.333^{c}		
K. pneumoniae ATCC 700603	2(200µg)	14.67 ± 0.882^{e}	12.00 ± 0.577^{d}	14.33 ± 0.882^{e}	$-^{a}$	18.33 ± 0.333^{f}
	1(100µg)	11.00 ± 0.577^{d}	8.00 ± 0.577^{c}	10.33 ± 0.333^{d}		
	0.5(50ug)	7.67 ± 0.333^{c}	5.33 ± 0.667^{b}	7.33 ± 0.667^{c}		
S. typhi ATCC 1912/R	2(200µg)	23.00 ± 0.577^{d}	$-^{a}$	$-^{a}$	$-^{a}$	30.00 ± 0.577^{e}
	1(100µg)	17.67 ± 0.333^{c}	$-^{a}$	$-^{a}$		
	0.5(50µg)	14.00 ± 0.577^{b}	$-^{a}$	$-^{a}$		

Note: Results are given as mean ± SEM of ZI (excluded the diameter of the well) in mm (n=3). ^{a,b,c,d,e,f,g,h} values of columns and rows marked(superscript) different letters for the same bacteria have statistical significance at $P\leq0.05$ (Duncan test).

- No zone of inhibition (for statically purpose 0 mm value was used)

ATCC: American type culture collection

In this study, mean ZIs of the same extract with each other at the same concentration on different bacteria statistically significant associations were determined. The results showed presence of different degrees of activities at different concentration of each extract against tested bacteria, at 2 mg/ml (200 µg) as described in Figure -4.

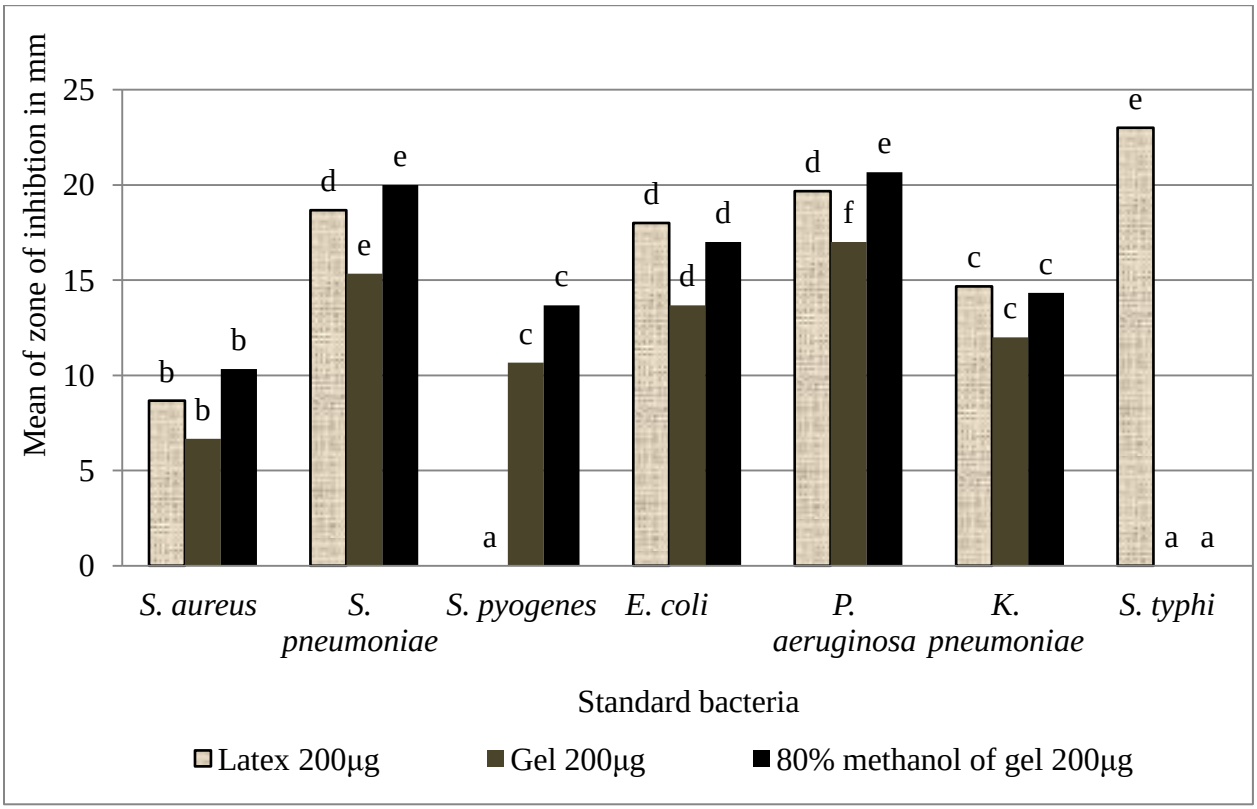

Figure - 4:- Statistical significances of each extract of the *Aloe macrocarpa* Tod leaf at 2 mg/ml on tested bacteria by mean of ZIs.

N.B. ZI values are means of triplicate determinations; values within the same color of bars followed by different letters superscripts are statistically significant (P≤0.05); i.e. different colors of bars do not share statistical significance.

The extracts that showed activity by diffusion method were further tested for MIC and MBC; the results are shown in Table -4. The MIC test results of the latex, hydroalcoholic gel, and crude gel ranged from 31.25- 4000, 31.25 - 2000 and 62.5- 8000 µg/ml, respectively. *S. aureus* was the least sensitive to all extracts with MIC values of 4000, 2000 and 8000 µg/ml for the latex, hydroalcoholic gel and crude gel, respectively. The lowest MBC was 31.25 µg/ml against *S. typhi* by latex. Each extract of *A. macrocarpa* Tod displayed bactericidal effect (MBC/MIC ≤4) on 83.33% (5/6) of the tested bacteria.

Table - 4:- Antibacterial activity (MIC and MBC in μg/ml) of the *Aloe macrocarpa* Tod extracts

Tested organisms	Effect types	Type of extract			Standard drug
		Latex	Gel	80% Methanol of gel	Ciprofloxacin
S. aureus	MIC	4000	8000	2000	5
ATCC 25923	MBC	32000	128000	16000	5
	MBC/MIC	8	16	8	1
S. pneumoniae	MIC	62.5	125	62.5	5
ATCC 49619	MBC	125	250	125	10
	MBC/MIC	2	2	2	2
S. pyogenes	MIC	NT	2000	1000	1.25
ATCC 19615	MBC	NT	4000	2000	2.5
	MBC/MIC	-	2	2	2
E. coli ATCC	MIC	31.25	500	62.5	1.25
25922	MBC	62.5	1000	125	1.25
	MBC/MIC	2	2	2	1
P. aeruginosa	MIC	31.25	62.5	31.25	1.25
ATCC 2706	MBC	62.5	250	62.5	2.5
	MBC/MIC	2	4	2	2
K. pneumoniae	MIC	500	2000	500	5
ATCC 700603	MBC	1000	4000	1000	5
	MBC/MIC	2	2	2	1
S. typhi ATCC	MIC	31.25	NT	NT	1.25
1912/R	MBC	31.25	NT	NT	1.25
	MBC/MIC	1	-	-	1

Note: NT = Not tested

4.4. Antioxidant activity

The antioxidant activities for all extracts as well as standard drug, the least %RSA was obtained at the lowest test concentration (1 μg/ml) while the highest %RSA was obtained at the highest test concentration (1000 μg/ml). The results obtained at the lowest concentration were 21.25±0.795, 9.41±0.445, 16.35±0.428, 27.33±0.363 and results at the highest concentration were 73.42±0.163, 67.38±0.195, 71.50±0.289, 88.03±0.318 for latex, crude gel, hydroalcoholic gel extracts, and ascorbic acid, respectively (Figure -5). IC_{50} of latex was the smallest (15.5 μg/ml) than gel extracts (Figure -6).

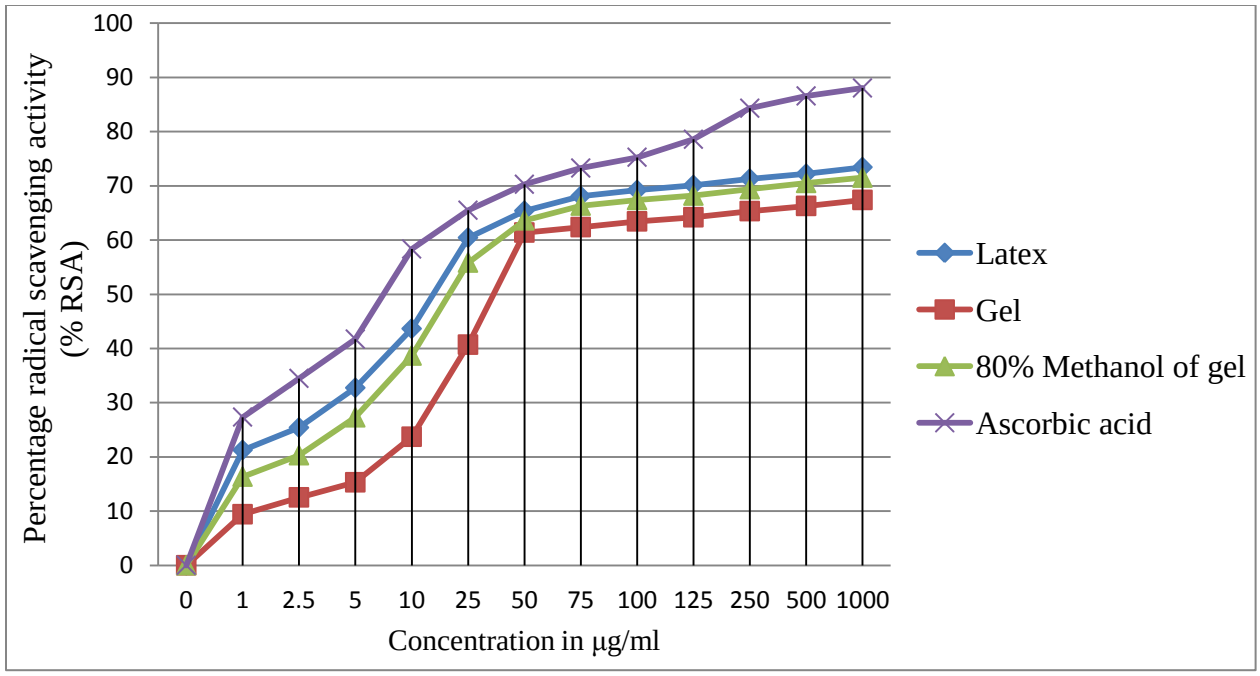

Figure - 5:- Percentage radical scavenging activities of the leaf of *Aloe macrocarpa* Tod extracts by DPPH method at different concentrations.

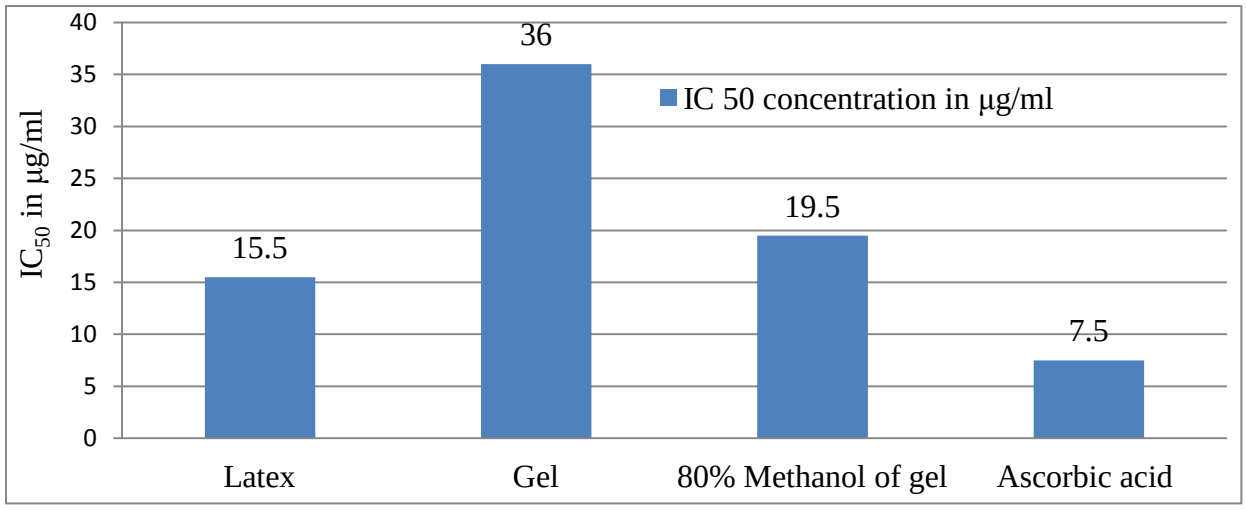

Figure - 6:- Experimentally determined IC_{50} values of the leaf of *Aloe macrocarpa* Tod extracts.

Antioxidant activities of the extracts were increased in a concentration dependant manner like ascorbic acid (positive control). However, at all tested doses of ascorbic acid between adjacent doses (the lower and higher tested doses) of p- values were statistically significant ($p<0.05$) except between 500 and 1000 µg/ml ($p>0.05$, $p= 0.196$).

However, both the latex and hydroalcoholic gel extracts were found statistically significant ($p<0.001$) from 1 up to75 μg/ml but above 75 μg/ml (75-1000 μg/ml) were statistically insignificant ($p>0.05$) between adjacent doses. For crude gel extract, statistically significant association ($p<0.001$) was found at doses ranging 1 - 50 μg/ml but not at higher doses. As compared with negative control (0 %RSA) all the three extracts at all tested doses were statistically significant ($p<0.001$).

The three extracts as compared to each other at proportional doses, latex and hydroalcoholic gel extracts showed higher antioxidant effects than crude gel, statistically significant ($p<0.001$). And latex also showed higher effects than hydroalcoholic gel, statistically significant ($p<0.05$).

4.5. Acute oral toxicity

No sign of toxicity or mortality was observed in mice after oral administration of the latex and gel of *A. macrocapa* Tod. This indicates that the oral LD_{50} is greater than 2000 mg/kg.

5. DISCUSSION

Plants are known to relieve various diseases including infectious and oxidative stresses, they are sources of large amount of drugs of different groups (69). In this finding leaf extracts of *A. macrocarpa* Tod have different degrees of antibacterial and antioxidant activities. Similarly, several studies have evaluated the effectiveness of traditionally used medicinal plants for treating bacteria and oxidative stress by different methods with various solvents extraction of leaves as well as other parts of plants (14, 17, 29, 30, 36). The variable antibacterial and antioxidant activities of the plant may be because of the existence of identity and concentration of secondary metabolites of plants responsible for antibacterial as well as antioxidant activities (9).

The leaf extraction of *A. macrocarpa* Tod showed different range of percentage yields and different drying time. This may be due to the difference in concentration and identity of secondary metabolites in the extracts (15). The 80% methanol gel extract was the highest yield (55.82%w/w) as compared to latex and crude gel; whereas latex revealed that higher percentage of yield (27.01%w/v) than crude gel (1.07%w/v) (Table -1). This may be because latex contains less amount of water than gel which is 98 - 99% water (only the active compound is 1 - 2%) in *Aloe* species (48). Hydro-alcoholic extract (80% methanol) dissolves many secondary metabolites of plants that have a wide variety of polar and moderately polar compounds (69) and most plants extracts by this solvent showed that antibacterial as well as antioxidant effects (14, 18, 29, 30). So, in this study, 80% methanol was used for extraction of gel based on the above justifications. According to this study, the absence of measurable yields by ethyl acetate and n-hexane from methanolic extraction residue of gel may be due to most active contents of gels may be solublized by 80% methanol.

Crude extracts have advantages from different perspectives like less-cost incurred and the possibility of developing phytopharmceuticals that cannot be isolated as a single active ingredient (30). The results of the preliminary phytochemical analyses of the three leaf extracts of *A. macrocarpa* Tod, possessed alkaloids, flavonoids, phenols, tannins, terpenoids and steroids while cardiac glycosides was absent; the latex extract contained anthraquinones and saponins in addition to the above phytoconstituents but not in gel extracts (Table -2). The presence of these phytoconstituents in the extracts may be responsible for the antibacterial and antioxidant activities of extracts (9, 69). This result is congruent with another previous phytochemical

analysis that revealed the presence of flavonoids in methanolic extract of the leaf of *A. macrocarpa* Tod (33). Though this study did not reveal the presence of anthraquinones and saponins in the gel extracts, their presence has been reported in the gel extracts of some other *Aloe* species (43, 45, 48, 49). This difference can be attributed to the difference in the species, geographical region, soil composition, season of collection and age of the plant (48, 56).

The three extracts of the plant showed various degrees of antibacterial activities against the tested bacterial species (Table -3 and Figure -4). As compared to negative control (DMSO) the antibacterial activities of the extracts were statistically significant ($p \leq 0.05$) except for latex against *S. aureus* at 0.5 mg/ml (1.33±0.577 mm) and *S. pyogenes* at all doses (no effect), for both gel extracts against *S. typhi* at all tested doses and for crude gel extract against *S. aureus* at 0.5 mg/ml. This statistical significance of the extracts may be further confirming presence of secondary metabolites in the extracts responsible for antibacterial activities. All extracts showed dose dependent responses which are statistically significant ($p \leq 0.05$) at tested doses (Table -3). Similarly, study done on methanolic extract of the stem bark of *Pteleopsis hylodendron* at 200, 100 and 50 mg/ml test doses showed statistically significant ($p \leq 0.05$) dose dependent antibacterial effect (29).

According to this study, latex showed the highest ZI 23.00±0.577 mm against *S. typhi* at 2 mg/ml, statistically significant ($p \leq 0.05$) (Figure -4). However, the gel extracts have no action against *S. typhi* (Table -3). This difference may be due to presences of saponins and anthraquinones in latex which were absent in gel extracts (Table -2) and these two metabolites are reported to have antibacterial activity (17, 36, 70). A study conducted on the latex of *A. harlana* showed a maximum ZI against *S. typhi* (Ty2) as compared to its effect on other test bacteria (39). The result of this study also corroborates the presence of promising substances in the latex of *Aloe* species for treatment of infections caused by this bacterium.

In this study, the latex was found to be ineffective against *S. pyognes* which contains all metabolites present in the gel extracts (Table -2). This may be due to presence of diverse sub chemical types in the extracts (9, 36). Additionally, methanol extract of *A. vera* gel exhibited least ZI from *S. typhi* (MTCC 531) statistically significant ($p<0.05$) as compared to its effect on other test bacteria (70); which didn't show activities by the gel extracts (Table -3). This

difference may be explained by nature of tested strains and phytoconstituents difference of the plants (6).

The gel extracts (crude gel and hydroalcoholic gel) of *A. macrocarpa* Tod, showed that there was no response difference against tested bacteria (Table -3 and 4). This may be attributed to presence of similar types of chemicals in the extracts (Table -2). However, ZIs of hydroalcoholic gel extract were greater than crude gel at the same bacteria and at proportional dose which are statistically significant ($p \leq 0.05$). These higher zone inhibitions of hydroalcoholic gel extract in all responded bacteria may be due to the presence of greater concentration of the active component(s) present in it than that in crude gel. This justification is supported by a study conducted on *A. vera* which is grouped under the same genus. When the concentration of the same individual ingredients of two extracts of *A. vera* were quantified and compared, it was found that the hydro-alcoholic (95% ethanol) gel extract was approximately 345 times more concentrated than that of the crude gel extract (36).

Dilution method is appropriate for assaying polar or non-polar extracts to determine MIC and MBC values (56). The MIC and MBC values of three extracts of *A. macrocarpa* Tod were further confirmed the presence of different degrees of antibacterial effects against tested bacteria between extracts and each extract with strain of bacteria; crude gel revealed highest values of MIC and MBC than latex and hydroalcholic gel against similar tested bacteria. Among Gram positive bacteria, *S. pneumoniae* was the most sensitive which had less MIC and MBC values for all extracts. MIC and MBC values for this organism were 62.5 and 125 µg/ml for both latex and hydroacoholic gel while 125 and 250 µg/ml for gel, respectively. Among Gram negative bacteria, *P. aeruginosa* was more sensitive for hydroalcoholic gel and crude gel which were 31.25/62.5 and 62.5/250 µg/ml with MIC/MBC values, respectively; latex showed less MIC values (31.5 µg/ml) for *E. coli*, *P. aeruginosa* and *S.typhi* but lowest MBC value obtained for *S. typhi* (31.5 µg/ml). This is again as mentioned above that increases the presence of more promising substance from latex extract against *S. typhi* (Table -4). Low MIC and MBC values indicate potentially high efficacy of the extracts as antibacterial agents (17, 29).

In agar well diffusion method ZIs of *S. pneumoniae* at 1 and 0.5 mg/ml were higher by hydroalcoholic gel extract than latex, statistically significant ($p \leq 0.05$) (Table -3) but MIC and MBC values (62.5 and 125 µg/ml, respectively) of both extract were similar (Table -4).

Additionally, ZIs of latex and hydroalcoholic gel extract on *E. coli* at all similar test doses were statistically insignificant ($p>0.05$) (Table -3) but MIC and MBC values of latex were less than hydroalcoholic gel extract (Table -4). These effects may be due to presence of non-polar substance in the latex extract that acts on both *S. pneumoniae* and *E. coli* bacteria. The diffusion method is not appropriate for testing non-polar samples or samples that do not diffuse easily into the agar (56).

According to Kognou, *et al*, 2011 and Okusa, *et al*, 2007 (14, 29), plant extracts could have two types of activities: bactericidal (MBC/MIC ≤4) and bacteriostatic (MBC/MIC > 4). Other important results that can be proven in this study depending on MIC and MBC values of extracts were presence of bactericidal activities (MBC/MIC ≤ 4) against both Gram positive and Gram negative tested bacteria by the three leaf extracts of *A. macrocarpa* Tod (Table -4). This may be one of the promising results for broad bactericidal effects of this plant and may be one of the reasons used in the community frequently for wound treatment (verbal communication). Each extract revealed bactericidal effect on 83.33% (5/6) of the tested bacteria. Bacteriostatic activity (MBC/MIC >4) was shown only against *S. aureus*, i.e., MBC/MIC was 8 for latex and hydroalcoholic gel and 16 for crude gel extract (Table -4).

In this study, antibacterial activities of the plant extract was found to be low compared to the standard drug (ciprofloxacin) except for *S. pneumoniae* by latex (14.33±0.882 mm) and hydroalcholic gel (17.00±0.577 mm) at 1 mg/ml; gel (15.33±0.667 mm) at 2 mg/ml of extracts were statistically insignificant ($p>0.5$). In addition, at 2 mg/ml of latex (18.67±0.667 mm) and hydroalcholic gel (20.00±0.577 mm) which were statistically significant ($p\leq0.05$) i.e. higher ZIs were found than ciprofloxacin (16.00±0.577 mm).

Generally, Gram negative bacteria are more resistant to antimicrobial agents compared with the Gram positive bacteria because they are covered with a phospholipid membrane carrying the structural lipopolysaccharide component that makes their cell wall impermeable to antimicrobial substances (13, 27). However, the present results revealed that the leaf extracts of *A. macrocarpa* Tod were active against both Gram positive and Gram negative bacteria, even lower MIC values were found in Gram negative bacteria for all tested extracts. These activities, therefore, could be explained from the chemical nature of the test samples, which might affect the overall impermeability and integrity of the bacterial cell wall (39, 69).

Oxidative stress appears to be involved in the development of several diseases (18). All of the three extracts were found to exhibit free radical scavenging properties in concentration dependent manner. As mentioned in the result, statistically significant association was observed between adjacent doses of each extracts at lower doses, i.e. % inhibition graphs of extracts characterized with nearly sharp increasing slope within concentration change of extracts (Figure - 5). This study is consistent with a study carried out in Ethiopia on *Clerodendrum myricoides*, *Satureja punctata*, and *Gnidia stenophylla* (18). Increasing the concentrations of the extracts revealed statistically insignificant association; these may be due to saturation. The reaction of antioxidants with DPPH is fast before steady state, since absorbance is directly proportional to the concentration of DPPH radicals (71).

Although the gel extracts showed various degrees of significant antioxidant effects, the latex revealed higher antioxidant effects than the gel extracts at proportional doses (Figure -5), this difference is statistically significant ($p<0.05$). Therefore, at similar doses antioxidant activities of latex were greater than hydroalcholic gel and crude gel whereas the crude gel was found to be the least (Figure -5). This is also confirmed by IC_{50} values which are 15.5, 19.5, and 36 μg/ml for the latex, hydroalcholic gel and gel extracts, respectively (Figure -6). The plant extracts with lower IC_{50} values have higher antioxidant activity and vice versa (18, 61). In this study, IC_{50} of latex extract was reveled to be more potent than the latex of *A. otallensis* (IC_{50} = 26.9 μg/ml) (40) but almost nearly equal value with the latex of *A. harlana* (IC_{50} = 14.21 μg/ml) (39).

The possible reasons for better antioxidant activity of latex than both gel extracts may be due to presences of saponins (9) and anthraquinones (18) which were absent in the later (Table -2). Additionally, during phytochemical analysis of phenolic compounds and tannins, early intense color was observed in the latex than gel and hydroalcoholic gel. These may be an indication of higher concentration existence of the above metabolite in latex.

In this study, higher %RSA were obtained by hydroalcholic gel than crude gel extract which were statistically significant ($p<0.001$) at all proportional concentrations. Similarly, study carried out on *A. vera* hydro-alcoholic (95% ethanol) gel revealed that higher antioxidant activities as compared to the gel, due to the presence of higher concentrations of total polyphenols from hydro-alcoholic gel extract than gel extract (36). Among natural antioxidants, plant polyphenols

are especially important because there is a correlation between total phenolic content and high antioxidant activity as well as reducing power of extracts (17).

In this study, the probable antioxidant mechanism of action of the extracts could be similar to that of ascorbic acid which prevents cell damage by binding to the free radical and neutralizing its unpaired electron (16) due to presence of polyphenoles. Polyphenolic compounds such as flavonoids, phenols and tannins are considered to be the major contributor to the antioxidant activity of medicinal plants. The antioxidants activities of polyphenols were attributed to their redox properties, which allow them to act as reducing agents, hydrogen donators, single oxygen quenchers and metal chelators (72). However, the mechanism of action of the extracts needs elucidation in order to ascertain the exact mechanism of action.

The acute oral toxicity tests were done for the two extracts, latex and crude gel, which are used directly for their antibacterial and antioxidant activities traditionally. The purpose of acute toxicity test was to detected presence or absence of toxicity in the standard way; because, toxicity is the main concern of indigenous therapeutic preparations (40). Sign of toxicity such as change in animal behaviour, lacrimation, weight loss, hair erection and mortality were not recorded and they were physically active for 14 days after plant extracts administration which fulfils the criteria set by OECD (68). Therefore, it can be said that the latex and gel are relatively safe for mice when given orally. These results showed safety of the extracts at the test concentrations and the traditional use of the plant material directly collected from the leaf of *A. macrocapa* Tod up to a dose of 2000 mg/kg by local people could be safe; however, species variation would also limit such a straight forward extrapolation of the findings of this study to humans.

Over all, the presence of wide variety chemicals and demonstration of good antibacterial activities against tested strains as well as antioxidant activities in different degrees by all of the tested extracts of *A. macrocarpa* Tod is an indication that the plant can serve as a useful source of chemical substances for the development of novel drugs.

6. STRENGTH OF THE STUDY

- The antibacterial and antioxidant tests were performed on the extracts of the plant (latex and gel) that are directly used by community and/or traditional healer.
- The tests were performed on three extracts of the plant and each test was done in triplicate to minimize sampling errors.
- The antibacterial activities were done in three ways (zone of inhibition, MIC and MBC).

7. LIMITATION OF THE STUDY

- The drying processes of the extracts were done by heat exposure.
- The antioxidant test was performed only by one method.

8. CONCLUSION

The result of this study revealed the presence of a wide range of phytochemicals in the extracts of the leaves of *Aloe macrocarpa* Tod with potential antioxidant, and antibacterial activities. From the results of acute toxicity of latex and gel, it can be concluded that the plant extract is relatively safe to mice; however, species variation would also limit such a straight forward extrapolation of the findings of this study to humans.

In both agar well diffusion and macro-broth dilution methods, all extracts displayed activities against both Gram negative and Gram positive bacteria. Thus, it can be conclude that extracts have broad spectrum activity against tested bacteria. Of all tested bacteria species, *S. typhi* was more sensitive for latex whereas *P. aeruginosa* for both crude gel and hydroalcoholic gel extracts.

All of the three extracts were also found to exhibit free radical scavenging activity in a concentration dependent manner especially at lower doses which are statically significant by DPPH assay. The latex was exhibited strongest antioxidant activity followed by, in descending order, hydroalcoholic gel and crude gel extracts.

From the results of this study, it can be concluded that the use of this plant in traditional medicine for the treatment of wound infections as well as chronic disease may be justified.

9. RECOMMENDATION

Based on the present study the following recommendations are proposed:

- The extracts should be investigated by other *in vitro* and *in vivo* models.
- Further studies should be conducted on other standard bacteria and any clinical isolated bacteria, even a greater range of organisms including fungi.
- The active constituent responsible for the reported activities should be elucidated.
- Further toxicological studies including sub- acute and chronic toxicity tests must be carried out.

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ANNEXS

PROCEDURES FOR PHYTOCHEMICAL ANALYSES

The qualitative phytochemical screening of active plant extracts of specific procedures were as follow:-

1. Alkaloids determination (63, 66)

Weighed 1 g of each extract and dissolving in 10 ml acid alcohol, boiled for 20 minutes at 100^{o}c in a water bath and filtered using Whatman filter paper. Taken 5 ml of the filtrate then added 2 ml 10% ammonia, and 5 ml chloroform then shaken gently. Extract the chloroform layer with 10 ml of 70% acetic acid and divided the solution in two portions in a test tube.

A. Mayer's test: Few drops of Mayer's reagent (Prepared by dissolving 1.36 g $HgCl_3$ in 60 ml distilled water and 5 g KI in 20 ml distilled water) were added in 3 ml test sample; creamy precipitate was observed that indicates presence of alkaloids.
B. Wagner's test: Few drops of Wagner's reagent (Prepared by dissolving 1.27 g of Iodine and 2 g KI in 100 ml distilled water) were added in 3 ml test sample; reddish brown color was observed that indicates presence of alkaloids.

2. Flavonoids determination(2)

About 10 ml of ethylacetate was added to 0.2 g of each extract and heated on a water bath for 3 minutes. The mixture were filtered, cooled and used for the following test.

A. Ammonium test: 3 ml of filtrate was shaken with 1 ml of dilute (10%) ammonium solution. The yellow colour in the ammonical layer indicates the presence of the flavonoids.
B. Aluminum chloride solution (1% test): another 3 ml portion of the filtrate was shaken with 1 ml of 1% aluminum chloride solution. The layers were allowed to separate; a yellow colour in the aluminum chloride indicates the presence of flavonoids.

3. Phenolic compounds determination

Weighed 0.2 g of each extract, dissolved in 5 ml of 95% ethanol then added 2 drops of 1M ferric chloride solution. Appearance of intense color indicates the presence of phenolic groups (2).

4.Tannins determination

Each extract of 0.5 g weighed, dissolved in 10 ml of water in at test tube and boiled then filtered. Few drops of 0.1% ferric chloride were added. Brownish green or a blue-black color develops that confirms the presence of tannins (63).

5. Terpenoids determination

Weighed 0.2 g of each extract then dissolved in 2 ml of distilled water and mixed with 2 ml of chloroform, concentrated (3 ml) H_2SO_4 was carefully added to form layer. A reddish brown coloration of the interface indicates the presence of terpenoids (64).

6. Saponins determination

Each extract of 0.2 g weighed and dissolved in 5 ml distilled water then shaken, heated until it boils. Frothing(appearance of creamy miss of small bubbles) shows presence of saponins (64).

7. Steroids determination

Weighed 0.2 g of each extract, dissolved in 10 ml chloroform and shaken to ensure dissolution, added 2 ml of 70% acetic acid to the solution cooled in refrigerator for 15 minutes and added 2 ml of concentrated sulfuric acid carefully. The color from violet to green in some samples indicates the presence of steroids (64, 65).

8. Anthraquinones determination

Weighed 1 g of each extract, dissolved in 10 ml of benzene and shaken, filtered with whatmann filter paper, added 5 ml of 10% ammonia solution to the filtrate and shaken. The presence of a pink or violet colour in the ammonical (lower) phase indicates the presence of free anthraquinones (17).

9. Cardiac glycoside determination

A 0.5 g of each extract was diluted in 5 ml of distilled water then shaken by adding 2 ml of glacial acetic acid and 1% of ferric chloride solution with few drops of concentrated H_2SO_4. A violet ring appears below the brown ring or a greenish ring form just above the brown ring and gradually spread throughout this layer which confirms presence of cardiac glycoside (63, 64).

DECLARATION

I hereby declare that the topic entitled "*In vitro* evaluation of antibacterial and antioxidant activities of the latex, gel, and 80% methanol gel extracts of the leaves of *Aloe macrocarpa* Tod (Aloaceae)" which is submitted to Department of Pharmacology, School of Pharmacy, College of Medicine and Health Sciences, University of Gondar in the partial fulfillment for the award of the degree of Master of Science in Pharmacology, is the result of work done by me in College of Medicine and Health Sciences, University of Gondar from December 1, 2013 to April 5, 2014. The activities were done in the laboratories of different departments; i.e. the extraction, phytochemical analyses, and antioxidant activities in pharmacognosy and pharmaceutical chemistry laboratory, acute oral toxicity test in pharmacology laboratory, and antibacterial test in microbiology laboratory under the guidance of Mohammedberhan Abdulwuhab (Assistant Professor of Pharmacology).

I further declare that the work is original and has not been submitted in part (or) full for the award of any other degree (or) diploma.

Name: Ashenafi Genanew

Signature: _____

Place: Gondar University

Submission Date: June 2014